

Immunotherapy with MVA-BN®-HER2 induces HER-2-specific Th1-immunity and alters the intratumoral balance of effector and regulatory T cells

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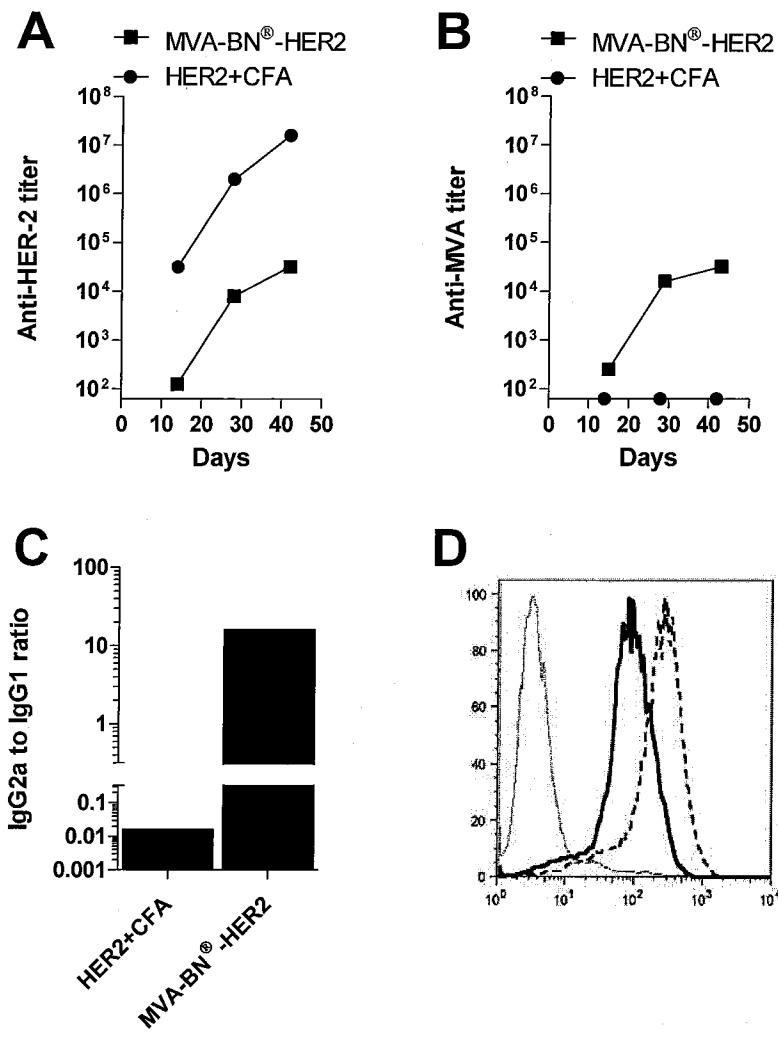
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Supplementary Fig. 1 MVA-BN®-HER2 induces potent Th1-dominated anti-HER-2 antibody responses.

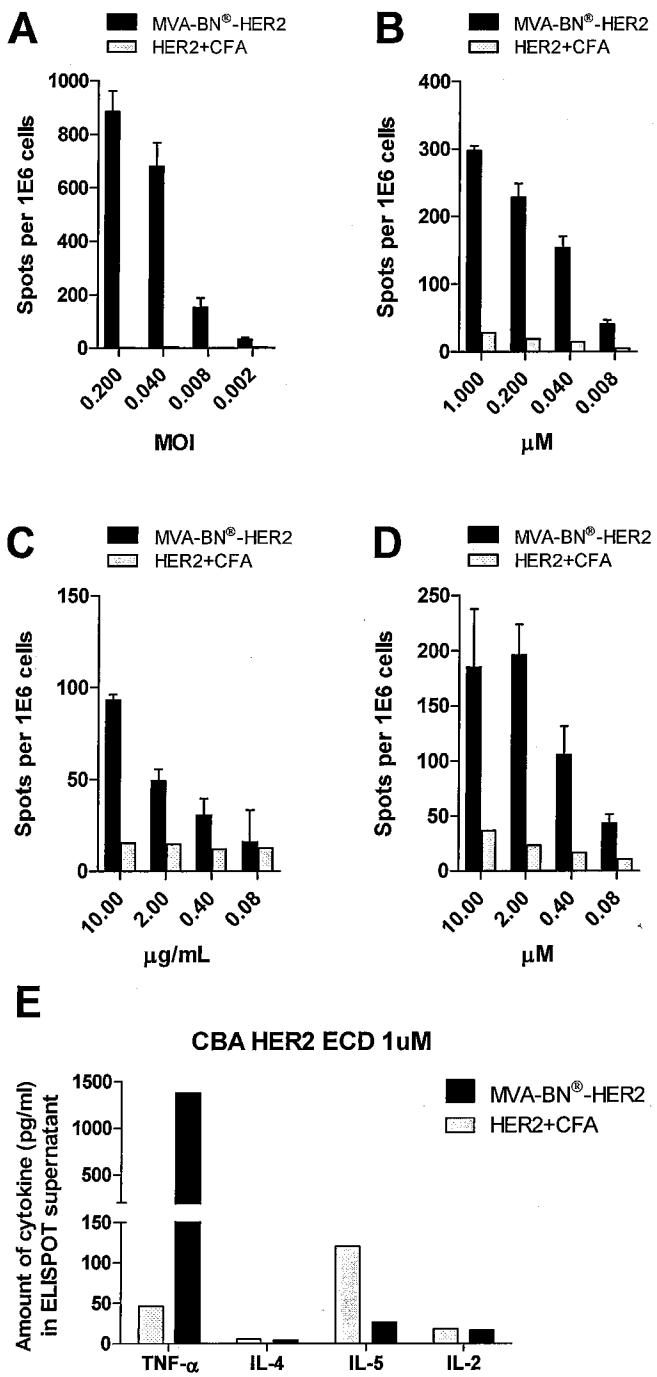
BALB/c mice were treated subcutaneously (s.c.) on days 1, 15, and 29 (q2wks x 3) with 5E7 TCID₅₀ of MVA-BN®-HER2, or 5 μ g of HER2 protein formulated in CFA, followed by two booster immunizations of 5 μ g HER2 protein formulated in Incomplete Freund's Adjuvant. Negative controls received Tris Buffered Saline (TBS). Serum was collected from all animals on days 14, 28, and 42, and HER-2 and MVA-specific antibody titers were evaluated by ELISA. **A** Anti-HER-2 antibody titers and **B** anti-MVA antibody titers in pooled sera from 5 mice per group treated either with MVA-BN®-HER2 (squares) or HER2+CFA (circles). **C** IgG2a / IgG1 isotype ratio of anti-HER-2 antibody titers **D** Antibody binding to human HER-2 expressed on CT26 tumor cells (serum at 1:100 dilution from MVA-BN®-HER2-treated animals (black solid line), HER2+CFA-treated animals (dotted line) or TBS-treated animals (grey line).

Treatment with MVA-BN[®]-HER2 induced HER-2- and MVA-specific antibody responses that increased with multiple administrations reaching a titer of 32,000 on day 42 for both antigens (Supplemental Figure 1A and 1B). Immunization with HER2+CFA induced higher titers than treatment with MVA-BN[®]-HER2 reaching a titer of 16,000,000 by day 42. Further analysis showed that both the kinetics and the overall magnitude of anti-HER-2 and anti-MVA titers are dose-dependent in animals treated with MVA-BN[®]-HER2 (data not shown).

To characterize putative adjuvant activities of the MVA-BN[®] vector on immune responses to the transgene, the ratio of anti-HER-2 IgG2a versus IgG1 antibody subtypes produced upon treatment with MVA-BN[®]-HER2 or HER2+CFA was determined. Immunization with MVA-BN[®]-HER2 induced a strongly Th1-dominated humoral response with an IgG2a/IgG1 ratio of 16 (titer of 4,000 for IgG1 and 64,000 for IgG2a, Supplemental Fig. 1C). Conversely, immunization with HER2+CFA induced a strikingly Th2-dominated antibody response with an antibody ratio of 0.016 (8,000,000 for IgG1 and 128,000 for IgG2a).

To address whether the anti-HER-2 antibodies induced by MVA-BN[®]-HER2 could bind to native HER-2 expressed on tumor cells, CT26-HER-2 cells were incubated with serum from mice treated with TBS, 5E7 TCID₅₀ MVA-BN[®]-HER2 or HER2+CFA. Sera of mice treated with either MVA-BN[®]-HER2 or HER2+CFA contained antibodies that bind cells expressing human HER-2 (Supplemental Fig. 1D).

Together, these results demonstrate that the MVA-BN[®] vector exerts potent Th1-biased adjuvant function towards the co-expressed HER-2-transgene resulting in the induction of strong, Th1-dominated, HER-2-specific antibody responses in mice treated with MVA-BN[®]-HER2. Treatment with HER2+CFA in contrast, induces strong Th2-dominated antibody responses.

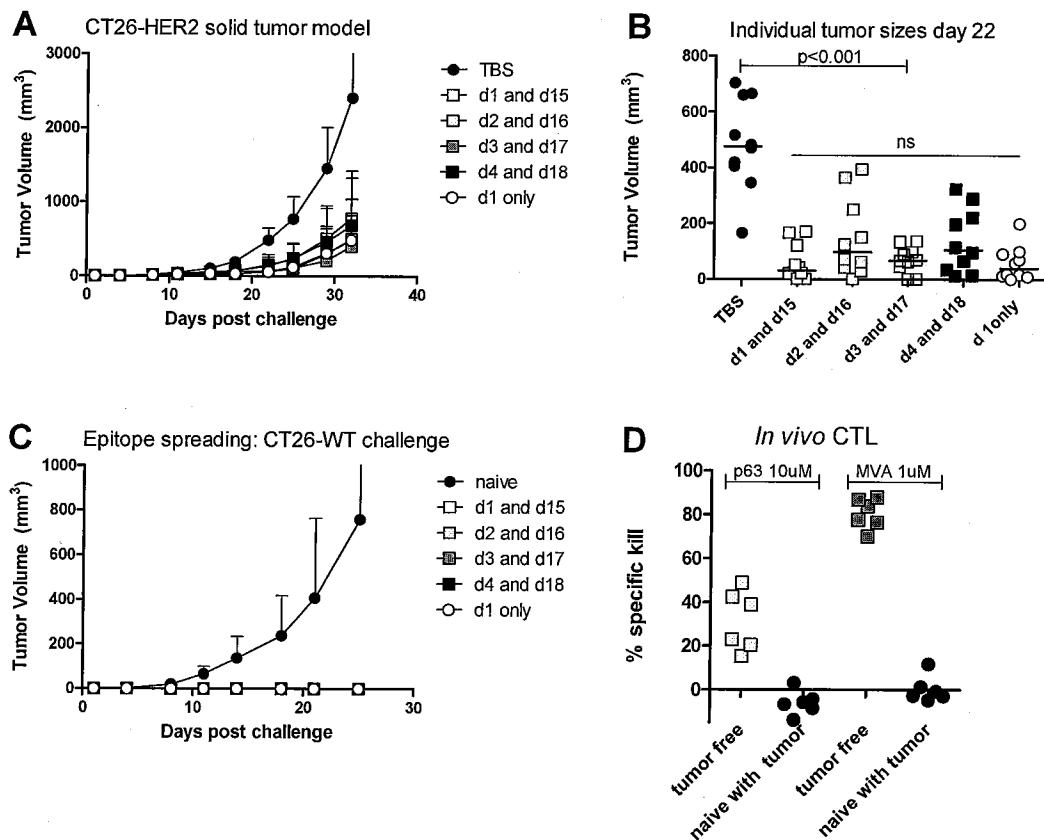


Supplementary Fig. 2 MVA- and HER-2-specific T cell responses in mice treated with MVA-BN®-HER2 or HER2+CFA.

Mice (3 mice/group) were treated on days 1, 15, 29, and 43 (q2wks x 4) with either MVA-BN®-HER or HER2+CFA. Seven days after the last treatment splenocytes from each treatment group were pooled and analyzed by IFN γ -ELISpot after restimulation with either: **A** MVA-BN®, **B** HER-2 ECD overlapping peptide library (166 overlapping 15mers), **C** HER-2 ECD protein, or **D** K^d-binding HER-2 peptide p63. **E** Cytokine profile in supernatants of wells restimulated with 1 μ M HER2 ECD OPL (panel B). For this analysis the “Mouse Th1/Th2 Cytokine kit” (a Cytometric Bead Array from BD, CAT.#551287) was used.

Strong MVA-specific T cell responses were detected in splenocytes of mice treated with MVA-BN®-HER2 reaching an average of 880 spots per million splenocytes (MOI 0.2). Under the same conditions only 2-4 IFN- γ spots per million cells were detected in HER2+CFA immunized mice (Supplemental Figure 2A) demonstrating the high specificity of this assay. More importantly, treatment with MVA-BN®-HER2 induced 5 to 10-fold higher frequencies of HER-2 specific T cells than HER2+CFA treatment, as seen by restimulation with 1 μ M of a HER-2 overlapping peptide library (HER-2 OPL) (297 spots versus 28, Fig. 2B) or with HER2 protein (Supplemental Figure 2C). While the addition of HER2 protein or HER2 OPL could potentially restimulate both CD4 and CD8 T cells, previous depletion experiments have shown that they only stimulate CD4 T cells to produce IFN- γ under the given ELISpot conditions (data not shown). Robust CD8-specific T cell responses were seen in MVA-BN®-HER2-treated mice upon restimulation with p63, a K^d-restricted HER-2 peptide, resulting in 5-fold higher T cell responses than observed after treatment with HER2+CFA (Supplemental Figure 2D).

Furthermore, large amounts of TNF- α (1378pg/ml) were found in pooled supernatants from splenocytes of MVA-BN®-HER2 treated animals that were restimulated with 1uM HER2 ECD OPL, while only marginal amounts of this cytokine was found under the same conditions in the splenocyte supernatants from HER2+CFA treated animals (46pg/ml). The reverse situation was found for IL-5. No difference was seen between treatment groups for IL-2 and IL-4 levels were below the detection limit. Together, these results demonstrate that MVA-BN®-HER2 induces potent Th1-dominated HER-2-specific cellular immune responses in mice treated with MVA-BN®-HER2 while treatment with HER2+CFA induces Th2 -dominated immune responses specific for HER-2.



Supplemental Figure 3: The CT26-HER2 solid tumor model.

A Tumor growth, 1E5 CT26-HER2 cells were injected intradermally (i.d.), tumors were measured twice weekly using calipers. Tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$. On day 32 all TBS-treated animals had to be taken down due to large tumor sizes. **B** Tumor volume of individual mice on day 22. Differences in tumor size at each particular day of tumor measurements were compared by One-Way ANOVA with Bonferroni's multiple comparison post-analysis. Differences compared to the TBS groups remained significant throughout the day the TBS mice had to be taken down (day 32). **C** On day 39 all tumor free mice (10/50 treated with MVA-BN®-HER2) were pooled and re-challenged with wild-type CT26 cells (1E5 cells, i.d.). As a control, 10 naïve mice were challenged with CT26 cells also (naïve). **D** 27 days after tumor challenge an *in vivo* CTL assay was performed on 6 tumor-bearing mice from the naïve group, 6 tumor-free mice of the previously MVA-BN®-HER2 treated animals that rejected both CT26-HER-2 and CT26-wt tumors, and 2 totally naïve mice to define background. Target cells (splenocytes) were labeled with either a 1 μ M mixture of 2 MVA epitopes (F2L and E3L) or with 10 μ M of p63 peptide. This assay was done about 4 weeks after the CT26-wt tumors were rejected, and 2 months since the last MVA-BN®-HER2 treatment, so the killing is primarily from a memory, and not effector phase, response.

In this experiment, 10 female BALB/c mice per group were implanted intradermally (i.d.) with 1E5 CT26-HER2 cells (the same cell line as used in the experimental pulmonary metastasis model)

and treatment was started on days indicated in the figure legend. The treatment schedule consisted of 2 vaccinations 2 weeks apart (q2wksx2). 1E7 TCID₅₀ of MVA-BN®-HER2 were given by skin scarification.

Significant anti-tumor efficacy was observed throughout the study with 20% tumor free mice by day 39 (10/50 across treatment groups). No difference was observed between groups in which treatment was initiated on day 1, 2, 3 or 4. Tumor free mice were re-challenged with CT26-wt tumor (which does not express HER-2). All mice completely rejected the challenge demonstrating that epitope-spreading to other tumor antigens had occurred. An *in vivo* CTL assay demonstrated that long-lasting CTL memory had been induced. 70-90% of MVA target cells labeled with a 1 μ M peptide mix of two MVA-dominant CTL epitopes were killed. In the same mice, 20-50% killing of p63 target cells was observed.

Together, these data demonstrate significant anti-tumor activity against i.d. implanted CT26-HER-2 cells that grow as solid tumors. Furthermore, epitope spreading and long-lasting immune memory was observed.